DISTRIBUTION AND OXIDATION OF MALONDIALDEHYDE IN MICE

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Abstract

The in vivo metabolism of malondialdehyde (MDA) by male and female Swiss mice was investigated. Distribution of an i.p. dose of MDA is rapid and uniform throughout the body. Conversion of ^{14}C-labeled MDA to CO_2 is complete 4 hours after an i.p. dose of 5 μmol to 200 μmol with no signs of short term toxicity. The yields of CO_2 from [1-^{14}C]-β-alanine, [3-^{14}C]-β-alanine, [1-^{14}C]-sodium acetate, and [2-^{14}C]-sodium acetate were also determined. Comparison of the yields of CO_2 from this series of compounds suggests the intermediacy of malonic semialdehyde in the metabolism of MDA. High doses (600 μmol) of β-alanine or acetate given prior to ^{14}C-MDA reduced the yield of $^{14}CO_2$. Ethanol and disulfiram were both inhibitors of MDA metabolism, indicating the involvement of aldehyde dehydrogenase in the oxidation of MDA.

These data demonstrate the ability of animal tissues to rapidly remove exogeneously administered MDA. They also have implications with respect to the possible pathological consequences of in vivo MDA generation.

Introduction

Malondialdehyde (MDA), a product of prostaglandin endoperoxide metabolism (1-4) and non-specific lipid peroxidation (5), is widely produced in mammalian tissues. It is unique among the endoperoxide metabolites in that its physiological function is unknown and its metabolic fate has been little studied. Crawford et. al. have shown that MDA is toxic to rats (6) and Basu and Marnett have established that it is mutagenic in *Salmonella typhimurium* (7). Studies by Yau indicate that MDA is cytotoxic and mutagenic when administered to a murine L5178Y lymphoma cell line cultured in vitro (8). The carcinogenic activity of MDA, or lack thereof, is a matter of some controversy (9,10).

Some time ago, Placer et. al. reported that the levels of thiobarbituric acid reactive material (product of a colorimetric reaction used for MDA estimation) in rat plasma declined rapidly after a single i.p. dose of MDA (11). More recently, Siu and Draper

reported that 60-70% of an oral dose of 65 nmol [1,3-^{14}C]-MDA was expired by rats as CO_2 in 12 hr (12). This confirmed the relatively rapid disappearance of MDA *in vivo* and indicated that a major pathway of its metabolism is oxidative. However, few animals were used in this study and no information was reported on tissue distribution, metabolic fate, etc. We have synthesized [1,2,3-^{14}C]-MDA and report here the results of our investigations of its uptake, distribution, half-life, and oxidation by mice. In addition, we have administered a number of potential intermediates of MDA metabolism to compare the extent of CO_2 evolution at each step of oxidation. Our results not only provide important new information about MDA metabolism but also offer critical insights regarding hypotheses that MDA is an important mediator of cellular pathology and transformation.

Materials and Methods

1,1,3,3-Tetramethoxy[1,3-^{14}C]-propane (TMP), [3-^{14}C]-β-alanine, [1-^{14}C]-sodium acetate and [2-^{14}C]-sodium malonate were purchased from Amersham. [1-^{14}C]β-alanine was from New England Nuclear. 1,1,3,3-Tetraethoxy[1,2,3-^{14}C]-propane was synthesized according to Bienkowski *et. al.* (13). The distribution of radioactivity in the latter compound is not uniform; carbons 1 and 3 contain 25% of the radioactivity each whereas carbon 2 contains 50%. [1,3-^{14}C]-Na-MDA and [1,2,3-^{14}C]Na-MDA were prepared by acid hydrolysis of the labeled tetraalkoxypropane precursor and purified by Sephadex LH-20 chromatography. Radiolabeled chemicals were mixed with unlabeled material to the final specific activities indicated: Na[1,3-^{14}C]-MDA (14 μCi/mmol), Na[1,2,3-^{14}C]-MDA (32.3 μCi/mmol), [1-^{14}C]-β-alanine (8.73 mCi/mmol), [3-^{14}C]-β-alanine (3 mCi/mmol), [1-^{14}C]-sodium acetate (0.95 mCi/mmol), [2-^{14}C]-sodium malonate (34 μCi/mmol). The carbon dioxide trapping agent CO_2met®, NCS tissue solubilizer, and PCS (liquid scintillation cocktail) were purchased from Amersham. The organ distribution of MDA was determined using a combustion apparatus kindly placed at our disposal at the Upjohn Company (Kalamazoo, MI).

Metabolism Experiments

Male and female Swiss albino mice were fed *ad libitum* before the experiment. Each compound was administered by intraperitoneal injection at a dose of 5 μmol or 100 μmol/mouse. Immediately after dosing, the mice were placed in metabolic cages and received no food or water during the course of the experiment. No difference in $^{14}CO_2$ evolution was detected when animals were allowed water. Expired $^{14}CO_2$ was trapped by pulling a slight vacuum on the metabolic cage and passing the air through two CO_2met® traps connected in series. Aliquots of 250 μl were periodically removed for counting. Urine and faeces were also collected and analyzed for radioactivity. Animals were sacrificed after each experiment, homogenized in a Polytron (Brinkmann) and digested with NCS tissue solubilizer (6 ml/g of tissue). Excess NCS was partially neutralized by adding 0.03 volume glacial acetic acid. ^{14}C was counted in PCS liquid scintillation cocktail after dark adapting overnight.

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Tissue Distribution of [^{14}C] MDA

[1,2,3-^{14}C]-MDA was administered by tail vein injection (50-100 mg/kg). The animals were sacrificed and tissues excised at 0.5, 2 and 5 hr. following injection. After adding cellulose (for even burning) and distilled water (for slow burning) the organs were placed in the combustion apparatus and the evolved CO_2 trapped and counted.

Inhibition of MDA Metabolism

To test the intermediacy of malonic semialdehyde in MDA metabolism, mice were dosed with 200 µmol or 600 µmol β-alanine followed immediately by 90 µmol of [1,3-^{14}C]-MDA. CO_2 expiration was determined as described. A similar experiment was performed with unlabeled acetate prior to [^{14}C]-MDA.

Inhibition of MDA metabolism was also studied by administering ethanol and/or disulfiram before injecting [^{14}C]-MDA. Ethanol (250 µL of 50% (v/v)) was injected i.p. 1 hour before MDA. Disulfiram was administered by gavage to animals that had been starved for 24 hr. MDA was injected 24 hours after disulfiram.

Results

Tissue Distribution

The distribution of radioactivity determined by combustion analysis of the organs of mice injected with [1,2,3-^{14}C]-MDA is shown in Table 1. Thirty minutes after injection the label is fairly evenly distributed throughout the organs. Two hours after treatment, the levels have begun to decline substantially in most organs. Kidney, liver, and adrenal remain elevated relative to the other tissues. At five hours post treatment the level of ^{14}C in the kidney and liver remains elevated. Persistent high levels in the bladder could be due to contamination with residual urine. The decline in ^{14}C levels in the various organs parallels the evolution of $^{14}CO_2$ from [1,2,3-^{14}C]-MDA (vide infra) which suggests oxidation of MDA is the major route for removal of injected MDA.

Table 1. Specific Radioactivity of Mouse Organs at Various Times Following Injection of [1,3-^{14}C]-MDA

Organ	Specific Radioactivity (dpm/mg ± S.D.) 0.5 hr	2 hr	5 hr
Spleen	24 ± 4	14 ± 2	11 ± 3
Heart	26 ± 4	12 ± 2	8 ± 2
Lung	28 ± 5	15 ± 2	13 ± 4
Adrenal	20	21 ± 5	12 ± 2
Kidney	27 ± 4	24 ± 7	19 ± 6
Bladder	37 ± 4	16 ± 4	20 ± 10
Uterus	28 ± 2	12 ± 2	10 ± 3
Fat pad	13 ± 6	6 ± 1	7 ± 3
Blood	22 ± 6	9 ± 1	7 ± 2
Stomach	20 ± 2	12 ± 2	9 ± 4
Thymus	19 ± 3	9 ± 1	8 ± 3
Brain	15 ± 2	7 ± 2	3 ± 2
Skin	19 ± 4	12 ± 3	10 ± 4
Muscle	15 ± 2	8 ± 2	6 ± 2
Bone	17 ± 1	9 ± 2	7 ± 3
Tail	23 ± 4	14 ± 5	8 ± 4
Intestine	22 ± 1	13 ± 2	11 ± 4
Liver	33 ± 1	22 ± 2	16 ± 2

(a) 10^6 DPM injected. Each point represents the average of 3 animals. ± Standard deviation

Metabolism to Carbon Dioxide

The time course for the evolution of $^{14}CO_2$ from [1,3-^{14}C]-MDA at various dose levels is shown in Figure 1. Initially there was rapid expiration of $^{14}CO_2$ which plateaued at about 2.5 hr then remained constant until 6 hr, the longest time point examined. The percentage conversion of MDA to CO_2 is relatively constant up to at least 90 μmol/mouse. A higher dose of 200 μmol/mouse was rapidly converted to CO_2 but some reduction in percent CO_2 expired was noted. The LD_{50} for MDA is about 400 μmol/mouse (2 g/kg, J. Buck, unpublished date). From the data in Figure 1 it is apparent the conversion of MDA to CO_2 is still substantial at half this value. The yield of CO_2 from MDA labeled only in the terminal carbons ([1,3-^{14}C]-MDA) is higher than that from [1,2,3-^{14}C]-MDA (Table 2). However, the time course for the evolution of CO_2 is similar in each case.

Possible intermediates in the metabolism of MDA include malonic semialdehyde and acetate. To evaluate the likelihood that these compounds are intermediates in MDA metabolism, we administered acetate and β-alanine labeled in specific positions with ^{14}C and compared the relative conversion of these compounds to CO_2 (Table 2). Pihl and Fritzson have shown that β-alanine is transaminated to

TABLE 2. Percent CO_2 Expired Four Hours After Injection of Labeled Compound[a]

[1,2,3-^{14}C] [1,3-^{14}C]- [2-^{14}C]- [1-^{14}C]- [3-^{14}C]-

No- Na- Ac- β-

TABLE 2. Percent CO_2 Expired Four Hours After Injection of Labeled Compound[a]

	[1,2,3-^{14}C] MDA	[1,3-^{14}C]- MDA	[2-^{14}C]- Malonate	[1-^{14}C]- Na-Acetate	[2-^{14}C]- Na-Acetate	[1-^{14}C]- β-Alanine	[3-^{14}C]- β-Alanine
Male Swiss Mice							
CO_2	52 ± 6[b]	75 ± 0.8	35 ± 2	81 ± 1	71 ± 1	89 ± 5	66 ± 4
Urine	4.2 ± 0.1	3 2	22 ± 22	2.9 ± 0.3	1 ± 9	6.1 ± 0.4	5.5 ± 0.8
Body	---- (c)	22 2	----	----	----	----	----
Female Swiss Mice							
CO_2	51 ± 3	----	31 ± 5	91 ± 8	70 ± 2	90 ± 5	74 ± 2
Urine	22 ± 4	----	51 ± 1	16 ± 16	2.9 ± 0.7	7 ± 2	9 ± 2
Body	24 ± 2	----	11 ± 3	11 ± 3	18.9 ± 0.6	2.8 ± 0.8	13 ± 1

(a) Data represent the average of at least 2 experiments ± standard deviations.

(b) Numbers represent percent of total injected radioactivity expired by 4 hours after treatment.

(c) ----, not determined.

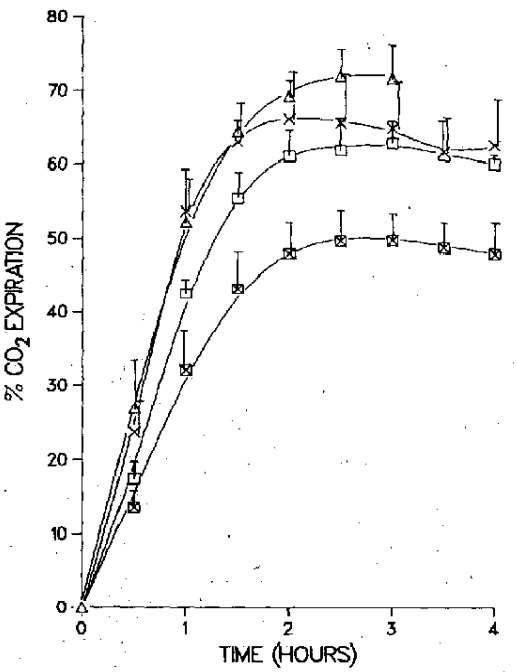


Fig. 1. Expiration of $^{14}CO_2$ from mice dosed with various amounts of malondialdehyde (% CO_2 expiration = {expired CPM/injected CPM} x 100%). Δ 5μmol, X 40 μmol, o 90 μmol, ⊠ 200 μmol.

malonic semialdehyde with very high efficiency (14). Therefore, we used β-alanine to generate malonic semialdehyde in vivo. Time courses of $^{14}CO_2$ evolution were measured for all the compounds. The values recorded in Table 2 are at four hours after treatment, which is when $^{14}CO_2$ expiration plateaued. No significant difference between males, females, starved, or fed animals was noted in the

rate or extent of CO_2 evolution. Similarily, no difference was noted between intravenous or intra-peritoneal administration of the compounds.

Carboxyl labeled sodium acetate gave 80-90% CO_2 within 1.5 hours of treatment and no further increases in CO_2 were detected. Acetate labeled in the methyl group gave 70% CO_2 and evolution was complete after 3 hours. The results with β-alanine were similar to those for acetate. Label in the carboxyl carbon was evolved as CO_2 in the first 1.5 hours whereas label at an internal position ([3-^{14}C]-β-alanine) gave lower yields of CO_2 (70%), which was evolved over a 3 hour period. The rapid release of CO_2 from both β-alanine and acetate indicates these compounds are rapidly oxidized. This is consistent with previous literature reports (14,17). The time course of CO_2 evolution from MDA as well as the extent of conversion is consistent with the intermediacy of malonic semialdehyde and acetate in the oxidative metabolism of MDA.

To further test the possible role of malonic semialdehyde and acetate in MDA metabolism, animals were treated with β-alanine (as a

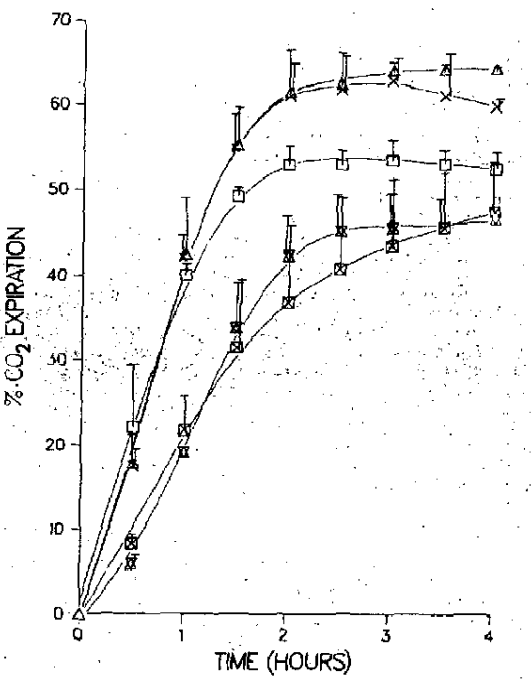


Figure 2. Expiration of $^{14}CO_2$ from mice treated with β-alanine or sodium acetate immediately prior to dosing with 90 μmol of [^{14}C]-MDA(% CO_2 expiration = [expired CPM/injected CPM] x 100%). X, 90 μmol MDA, Δ 200 μmol β-alanine + [^{14}C]-MDA, XX 600 μmol β-alanine + [^{14}C]-MDA, □ 200 μmol sodium acetate + [^{14}C]-MDA, ⊠ 600 μmol sodium acetate + [^{14}C]-MDA.

source of malonic semialdehyde) or acetate (ph 7.0) prior to dosing with 90 µmol of [^{14}C]-MDA. The effect of these compounds on $^{14}CO_2$ expiration is shown in figure 2. There was no effect of β-alanine at the 200 µmol dose whereas 200 µmol of acetate was slightly inhibitory. Treatment with 600 µmol of either compound resulted in a definate decrease in $^{14}CO_2$ expiration. These data suggest that high levels of malonic semialdehyde or acetate can compete with the metabolism of MDA.

We administered [2-^{14}C]-malonate to mice in an attempt to assess the possible intermediacy of malonate in the metabolism of MDA. There was relatively little CO_2 evolution and large amounts of ^{14}C were detected in the urine of these mice. This is consistent with inefficient distribution of malonate to the various tissues and excretion via the kidneys. These observations preclude an accurate evaluation of the possible intermediacy of malonate in MDA metabolism.

Alternate Routes of Excretion and Metabolism

Significant amounts of radioactivity were found in the urine of all animals dosed with any of the compounds. The highest percentages of urinary radioactivity were seen in those mice treated with sodium malonate. The variability in the urinary radioactivity is due in large part to different amounts of urine collected over the course of the four hour experiments.

Urine collected from the bladders of mice dosed with [1,2,3-^{14}C]-MDA was analyzed by HPLC to see if the radioactivity was due to unreacted MDA. None of the radioactivity in urine coeluted with MDA. Authentic ^{14}C-MDA is stable in urine for several hours. Therefore, the radioactive compounds are metabolites of MDA and not the products of reaction of MDA with a urinary constituent.

To determine if volatile metabolites of MDA, other than CO_2, were released in expired air, a trap containing ethyl acetate cooled by dry ice-acetone was placed after the CO_2 traps. No radioactivity was detected, indicating the absence of volatile metabolites.

The amount of ^{14}C from MDA incorporated into lipids of the mouse carcass was estimated by extraction with chloroform: methanol (2:1). The extract was concentrated and loaded onto a preparatory TLC plate of silica gel G. The plate was developed in hexane :diethyl ether: acetic acid (160:50:3) to separate free cholesterol, glycerides, and phospholipids. Four hours after administration about 20% of the ^{14}C in the carcass was found in the lipid extract and about 25% of this was associated with free cholesterol. This suggests that MDA is incorporated into the metabolic pool of acetate.

Inhibition of MDA Metabolism

Inhibitors of aldehyde dehydrogenase were tested for their effect on the metabolism of MDA. Disulfiram is an inhibitor of aldehyde dehydrogenase whereas ethanol is oxidized to acetaldehyde which could function as a competitive inhibitor of MDA metabolism. The effect of pretreatment of the animals with either disulfiram or ethanol on the metabolism of [1,2,3-^{14}C]-MDA is shown in Table 3.

Administration of ethanol (5 mmol/mouse) inhibited CO_2 expiration by 45% whereas disulfiram (134 μmol/mouse) decreased CO_2 expiration by 73%. A lower dose of disulfiram was ineffective. Administration of both inhibitors together did not inhibit metabolism any more than the higher dose of disulfiram alone.

TABLE 3. Effects of Ethanol and Disulfiram on CO_2 from [1,2,3-^{14}C]-MDA

Pretreatment	% CO_2
none	52 ± 6
5.1 mmol ethanol[a]	29 ± 3
44 μmol disulfiram[b]	48
134 μmol disulfiram	14. ± 5
ethanol + disulfiram (134 μmol)	16.8

a: i.p. injection 1 hour before MDA.
b: p.o. 24 hr to mice starved 24 hrs prior to MDA
c: data represent the average of at least 2 experiments ± S.D. except for 44 μmol disulfiram and ethanol plus disulfiram which were single determinations.

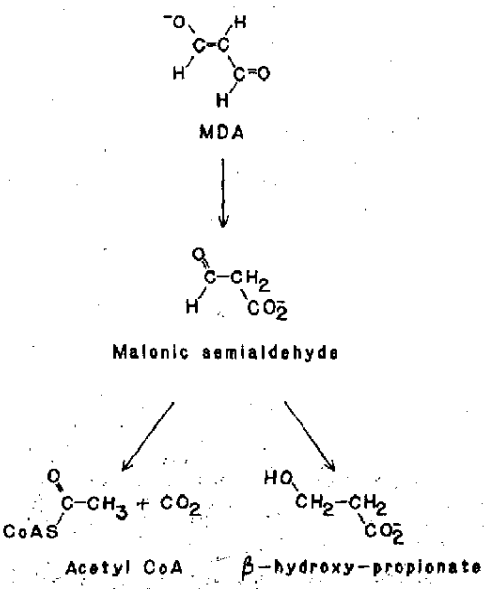
Discussion

The present studies were undertaken to investigate the metabolism of MDA, in Swiss mice, a strain in which MDA has been reported to be tumorigenic (9). MDA is quickly and uniformly distributed to all tissues. It is rapidly incorporated into pathways of intermediary metabolism. A majority of the metabolic pool of MDA is combusted to CO_2 but some enters acetate-requiring biosynthetic pathways. The amounts of metabolites in urine are variable but urine does not contain any unreacted MDA.

One possible pathway for metabolism of MDA is given in Scheme I. In this scheme, MDA is oxidized to malonic semialdehyde which is then oxidatively decarboxylated to yield CO_2 and acetyl CoA. The acetyl CoA can then enter the TCA cycle to yield 2 more molecules of CO_2.

The metabolism of β-alanine provides a clue to the extent of metabolism which could occur via this path. Pihl and Fritzson have suggested that β-alanine, which is converted to CO_2 in high yield, is first transaminated to malonic semialdehyde which is then rapidly oxidized to CO_2(14). Therefore, based on the yield of CO_2 from β-alanine and sodium acetate one can calculate that metabolism of MDA, via the intermediate malonic semialdehyde, should yield 80% CO_2 from [1,3-^{14}C]-MDA and 75% CO_2 from [1,2,3-^{14}C]-MDA. The internal carbon of [1,2,3-^{14}C]-MDA has twice the specific activity of the terminal carbons, i.e., half the ^{14}C is in the 2 carbon and the other half is divided between the terminal positions. The 2 carbon of MDA would yield the methyl carbon of acetate, which is less readily metabolized to CO_2 than the carboxyl carbon. Therefore the

theoretical yield from [1,2,3-^{14}C]-MDA is lower than that from MDA labeled only in the terminal carbon.



Scheme 1. Possible pathway of MDA metabolism involving initial production of malonic semialdehyde.

There are several possible reasons for the lower than theoretical yield of CO_2 obtained from ^{14}C-MDA. MDA is known to bind to proteins and nucleic acids(15,16). It is likely that a portion of the administered dose does not get metabolized but becomes bound to macromolecules upon injection. A second possibility is that conversion of MDA to MSA is not quantitative which would lower the yield from that calculated on the basis of β-alanine combustion.

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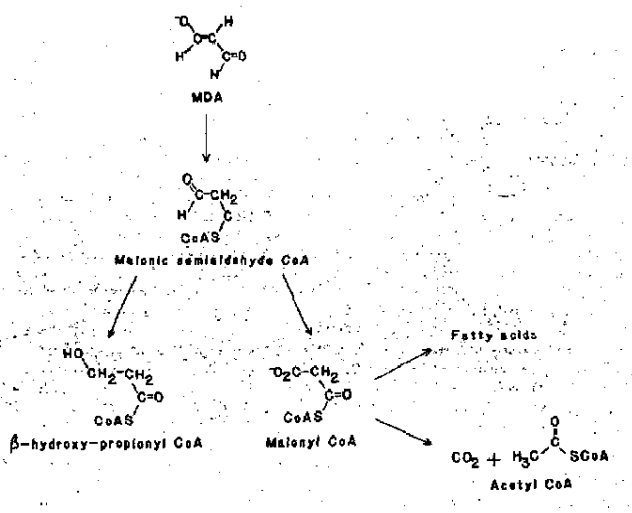
The inhibition of MDA metabolism by treatment of the mice with β-alanine and acetate lend support to this proposed pathway. The doses required to achieve inhibition were rather high, but this is reasonable considering the high metabolic capacity of mice for all three compounds. There were no adverse effects of these treatments on the appearance of the animals during the course of the experiments.

An alternate route for metabolism of MDA is presented in Scheme II. The key intermediate in this pathway is malonate, which is esterified with coenzyme A to yield malonyl CoA. The malonyl CoA could then be metabolized to CO_2 and acetyl CoA. Incorporation of malonyl CoA into fatty acid biosynthesis is also possible. Conversion of [2-^{14}C]-malonate to CO_2 was relatively low and if this pathway is operative one calculates that 28% of ^{14}C from [1,2,3-^{14}C-]MDA should arise as CO_2. Since the yield of CO_2 from MDA is greater than this it is unlikely the pathway is important. However,



Scheme 2. Possible pathway of MDA metabolism involving initial production of malonic semialdehyde CoA.

the inefficient transport of externally administered malonate into cells makes this conclusion uncertain(17).

The effects of disulfiram and ethanol on CO_2 expiration suggest the involvement of aldehyde dehydrogenase in MDA metabolism. Both compounds inhibit but do not act in a synergistic or additive fashion. Sugimoto has described a cytoplasmic aldehyde dehydrogenase which utilizes MDA as a substrate (18). Mitochondrial and microsomal dehydrogenases that will accept MDA as a substrate have also been described(18).

MDA is commonly assayed by a colorimetric assay with thiobarbituric acid (TBA). Using this assay, MDA concentrations of 1-3 μM are reproducibly detected in human blood (19). The fact that low and high dose MDA is rapidly and completely metabolized in mice and the observation that no MDA is detected in urine of treated animals makes it unlikely that ambient concentrations of 1-3 μM MDA are present in plasma. This suggests that the pigment observed in the TBA assay does not arise via reaction with MDA. Yagi has suggested that lipid peroxides are responsible for the TBA-reactive material in plasma (20). However, a new assay for lipid hydroperoxides demonstrates that the TBA assay overestimates plasma lipid hydroperoxide concentration approximately one hundred-fold (19). In addition, no correlation is observed between actual lipid peroxide concentrations and levels of TBA-reactive material in plasma (19). Thus, the identity of the TBA-reactive material in plasma is unknown.

Recent work from our laboratory has verified that MDA is mutagenic to Salmonella , although weakly (7). Topical application of the same material to mouse skin, however, provides no evidence for tumor initiating, promoting, or complete carcinogenic activity (10). Considering the high rate of metabolism of MDA, these findings suggest that the carcinogenic risk associated with, for example, vascular MDA synthesis is minimal. However, this may not be the case in peripheral tissue or under conditions where MDA metabolism is inhibited.

Acknowledgements

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ABSTRACT

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INTRODUCTION

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Oxytocinase is known
serum, placenta and u
tive inhibition of t
cyclic GMP (cGMP) wit
inhibition at pH 7.4 (
it appears that part o
ed to their ability
protecting endogenous
in parturition partly